

## Identification and Characterization of an Immunodominant 28-Kilodalton *Coxiella burnetii* Outer Membrane Protein Specific to Isolates Associated with Acute Disease

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*Coxiella burnetii* causes acute Q fever in humans and occasional chronic infections that typically manifest as endocarditis or hepatitis. Isolates associated with acute disease were found to be distinct from a group of chronic disease isolates by a variety of biochemical parameters and in a guinea pig fever model of acute disease, suggesting a difference in virulence potential. We compared antigenic polypeptides among *C. burnetii* isolates and found an immunodominant 28-kDa protein in acute group isolates but not in chronic group isolates (T. Ho, A. Hotta, G. Q. Zhang, S. V. Nguyen, M. Ogawa, T. Yamaguchi, H. Fukushi, and K. Hirai, Microbiol. Immunol. 42:81-85, 1998). In order to clone the *adaA* gene, the N-terminal amino acid sequence of *adaA* was determined and a 59-bp fragment was amplified from Nine Mile phase I DNA by PCR. The putative gene fragment was used to screen a lambda ZAP II genomic DNA library, and an open reading frame expressing a 28-kDa immunoreactive protein was identified. Sequence analysis predicted a gene encoding an ~28-kDa mature protein with a typical signal sequence. The *adaA* (acute disease antigen A) gene was detected in acute group *C. burnetii* isolates but not identified in chronic group isolates by PCR and Southern blotting. A typical signal peptide was predicted in *adaA*, and specific antibody to *adaA* reacted with the purified membrane fraction of acute group isolates by Western blotting, suggesting that *adaA* is exposed on the outer surface of *C. burnetii*. *adaA* was overexpressed in pET23a as a fusion protein in *Escherichia coli* to develop anti-recombinant *adaA* (anti-*radaA*) specific antibody, which recognized a ~28-kDa band in acute group isolates but not in chronic group isolates. In addition, immunoblotting indicates that *radaA* reacted with sera derived from animals infected with acute group isolates but did not react with sera from animals infected with chronic group isolates. These results support the idea that an *adaA* gene-targeted PCR assay and an *radaA* antigen-based serodiagnostic test may be useful for differential diagnosis of acute and chronic Q fever.

*Coxiella burnetii* is an obligate intracellular bacterium that causes acute and chronic forms of Q fever in humans. Acute Q fever is an influenza-like illness that usually is self-limiting and effectively treated by antibiotics (11). In contrast, chronic Q fever is a severe, sometimes fatal disease, and patients have responded poorly to various antibiotics (8, 20). Endocarditis is the most common chronic manifestation, while vascular infection, bone infection, and chronic hepatitis are also reported (21). Infection in most animals is mainly subclinical, but abortion and infertility are common manifestations in ruminants (2). Domestic animals, especially cattle, sheep, and goats, are important reservoirs of the agent responsible for infection of humans (7, 11).

*C. burnetii* has been isolated from various sources including milk, ticks, and humans with acute and chronic Q fever worldwide (2, 7, 8, 10). Previous studies have demonstrated that *C. burnetii* isolates originating from milk, ticks, and humans with

acute Q fever differ in plasmid type (22), lipopolysaccharide profiles (3), and chromosomal DNA restriction endonuclease fragment patterns (5) from many isolates originating from chronic Q fever. The differences at the phenotypic and molecular levels between acute and chronic disease-associated isolates suggested that there may be a virulence potential characteristic of each group of isolates. Samuel et al. first reported that *C. burnetii* isolates associated with acute Q fever contained the QpH1 plasmid, while isolates associated with chronic Q fever possessed the QpRS plasmid or the plasmid sequences were integrated into the chromosome (22, 23). More recent studies of several *C. burnetii* isolates from Europe detected either the QpH1 plasmid-specific sequences (25, 26) or plasmid type QpDV (27) in both acute and chronic disease-associated isolates, suggesting that there was no specific gene(s) on plasmids responsible for a specific virulence phenotype. These data supported the notion that chronic disease could result from isolates associated with acute disease and might result from unique patient factors associated with immune status (25–27). However, no chronic disease-associated organisms have been isolated from acute Q fever patients. Therefore, it is quite possible that there are bacterial genetic factors responsible for acute disease. This hypothesis was supported in a

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TABLE 1. Original source, pathogenic characteristics, genetic group, and plasmid type of *C. burnetii* strains

Group <sup>a</sup>	Plasmid type <sup>b</sup>	Isolate <sup>c</sup>	Phase	Original source	Disease or type	Passage <sup>d</sup>
I	QpH1	Nine Mile RSA493	I	Montana, tick, 1935		307GP/1TC/1EP
		Turkey RSA333	II	Turkey, human blood, 1948	Acute	31EP
		African RSA334 (> <sup>e</sup> )	I	Central Africa, human blood, 1949	Acute, Congolese red fever	3HP/4EP
		Giroud RSA431 (> <sup>e</sup> )	I	Central Africa, human blood, 1949	Acute, Congolese red fever	2GP/2EP
		El Tayeb RSA342	I	Egypt, tick, 1967		4GP/2EP
		Panama RSA335	I	Panama, chiggers, 1961		4EP
		California 33 RSA329	I	California, cow's milk, 1947	Persistent	6EP
		Ohio 314 RSA270	I	Ohio, cow's milk, 1956	Persistent	4EP
		Henzerling RSA331	II	Italy, human blood, 1945	Acute	36EP
		Priscilla	I	Montana, goat cotyledon, 1980	Abortion	GP/2EP
II	QpRS	KQ154	I	Oregon, human heart valve, 1976	Endocarditis	HV/2EP
IV		GQ212	I	Nova Scotia, Canada, human heart valve, 1981	Endocarditis	HV/2EP
V	NP	SO217	I	Montana, human liver biopsy sample, 1981	Hepatitis	BX/2EP
		KoQ229	I	Nova Scotia, Canada, human heart valve, 1982	Endocarditis	HV/2EP
		Dugway 7E22-57	I	Utah, rodents, 1958		3EP
VI	QpDG	MAN	I	French, human blood	Aortic aneurysm	?
— <sup>f</sup>	QpDV	ME	I	French, human heart valve	Endocarditis	?

<sup>a</sup> As defined by restriction enzyme banding patterns (5).

<sup>b</sup> Plasmids were described elsewhere (22, 27).

<sup>c</sup> Provided by Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Mont. Reference strains were determined by complement block titration (M. G. Peacock, Rocky Mountain Laboratories).

<sup>d</sup> Numbers indicate passage number; GP, guinea pig passage; TC, tissue culture; EP, egg passage; HP, hamster passage; HV, heart valve; BX, liver biopsy sample; ?, passage prior to receipt in authors' laboratory not known.

<sup>e</sup> >, passage history variants.

<sup>f</sup> —, MAN and ME were not classified (27).

study by Moos and Hackstadt (17) comparing virulence of a prototype isolate from each group in guinea pigs. The acute disease group prototype isolate (Nine Mile phase I RSA493) caused infection and fever when delivered intraperitoneally with less than 10 organisms, while the chronic disease group prototype isolate (Priscilla Q177) required at least 10<sup>5</sup> organisms to cause fever.

Our previous study identified a 28-kDa protein (P28) that was immunodominant in isolates originating from milk, ticks, and humans with acute Q fever but not immunogenic in isolates originating from chronic Q fever (6). This finding suggested that *adaA* could be associated with a pathogenic factor of acute Q fever. *adaA* may also have value as a marker to distinguish isolate groups. In order to clone and characterize the *adaA*-encoding gene, the N-terminal amino acid sequence of the protein was determined by protein sequencing. A 59-bp gene fragment was amplified from Nine Mile phase I DNA by PCR with one primer pair designed based on the N-terminal amino acid sequence and was used as a probe to screen a genomic library by Southern hybridization. The gene encoding P28 was cloned and sequenced. Outer membrane localization and antigenicity of *adaA* indicated that *adaA* may be a virulence factor related to acute Q fever, and the *adaA* gene may be a useful genetic marker for differentiation of isolates of *C. burnetii*.

#### MATERIALS AND METHODS

**Bacterial strains, phage, and growth conditions.** Seventeen *C. burnetii* isolates from various clinical and geographical sources were used in this study. The original source, pathogenic characteristics, and genetic properties of these strains are summarized in Table 1. All the isolates were propagated in BGM or L929 cell cultures and purified as described elsewhere (7, 22). The bacteriophage lambda ZAP II (Stratagene, La Jolla, Calif.) was used as the vector for construction of

the *C. burnetii* expression genomic DNA library. *Escherichia coli* XL-Blue MRF<sup>+</sup> (Stratagene) was cultured in Luria broth (LB) with 12.5 µg of tetracycline/ml and used as the host strain for recombinant plasmids and bacteriophage lambda ZAP II.

**Preparation of *C. burnetii* OMPs.** The outer membrane proteins (OMPs) of *C. burnetii* were extracted from purified *C. burnetii* Nine Mile based on the method described by Ohashi et al. (19). Briefly, purified organisms were suspended in 10 mM sodium phosphate buffer, pH 7.4, containing 1% Sarkosyl (Sigma, St. Louis, Mo.) and 50 µg each of DNase I and RNase A and incubated at 37°C for 30 min. EDTA at a final concentration of 15 mM was added to stop the nuclease reaction. The insoluble precipitates were obtained by centrifugation at 10,000 × g for 1 h, washed twice with 0.1% Sarkosyl-phosphate-buffered saline, and then resuspended in STE buffer (100 mM NaCl, 10 mM Tris-HCl, and 1 mM EDTA, pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride (Sigma).

**Analysis of the N-terminal amino acid sequences of *adaA*.** The OMPs of *C. burnetii* Nine Mile were separated by reversed discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to a polyvinylidene difluoride membrane as described elsewhere (19). The presence of *adaA* in the purified membrane fraction of *C. burnetii* Nine Mile was confirmed by immunoblotting as described previously (6). The portion of the polyvinylidene difluoride membrane containing *adaA* was excised and analyzed with the HP G1005A protein sequencing system (Takara Shuzo Co., Kyoto, Japan).

**Preparation of DNA probe specific to the P28-encoding gene.** The N-terminal amino acid sequence of *adaA* was determined as ENRPILNTINYQQQVEKWV TTDSADVMVSVN. Based on the N-terminal amino acid sequence, a pair of primers, P28a (5'-ATHAAYTAYCARCARGT-3') and P28b (5'-AGCAT NACRTCNGC-3'), were designed and used to amplify a 59-bp fragment of the putative *adaA* gene. The expected 59-bp product was amplified from *C. burnetii* Nine Mile DNA by PCR with these primers. The nucleotide sequence of the 59-bp fragment was determined by the dideoxy nucleotide chain-termination method as described previously (29). Sequence analysis of the 59-bp fragment indicates that the deduced amino acid sequence is identical to the chemically determined N-terminal amino acid sequence of P28, suggesting that the 59-bp fragment is specific DNA of the *adaA* gene. Based on the determined nucleotide sequence, specific primers P28a1 (5'-ATTAATTATCAACAGCAGGTTG-3') and P28b1 (5'-AGCATTACATCGGCAGAAATCC-3') were designed and used to amplify the 59-bp specific fragment of the *adaA* gene from *C. burnetii* Nine Mile DNA. The amplified 59-bp fragment was labeled by the random primer

extension method with the digoxigenin DNA labeling kit (Roche Diagnostics K. K., Tokyo, Japan) and used as a DNA probe to screen the genomic DNA library of *C. burnetii* by Southern hybridization.

**Construction and screening of genomic DNA library.** A lambda ZAP II genomic DNA library was constructed as described by Macellaro et al. (9) and screened by Southern hybridization with the *adaA* gene-specific probe. Briefly, the genomic library was plated on *E. coli* XL-Blue MRF' to yield about 500 plaques per plate. Plates were incubated at 37°C until plaques were 1 mm in diameter. Plaques were transferred onto a nylon membrane (Amersham Pharmacia Biotech, Piscataway, N.J.) and were hybridized with the *adaA* gene-specific probe according to the protocol provided by the manufacturer (Roche Diagnostics K. K.). The positive plaques were detected by using the digoxigenin luminescent detection kit (Roche Diagnostics K. K.). In vivo excision of the pBlue-script vector along with the inserted DNA of each positive clone was performed according to the protocol of the supplier of the lambda ZAP II cloning system.

**Immunoblot analysis of *adaA* expression in *E. coli*.** *E. coli* containing the recombinant plasmid was cultured in LB supplemented with 4 mM IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) at 37°C overnight, and then cells were pelleted by centrifugation. The cell pellet was analyzed by SDS-PAGE and immunoblotting with rabbit anti-Nine Mile serum as described previously (30).

**DNA sequence analysis.** Plasmid DNAs from positive clones that expressed immunoreactive protein were isolated and purified by using the FlexyPrep kit (Amersham Pharmacia Biotech). The nucleotide sequence was partially determined by the dideoxy nucleotide chain-termination method with the Thermo Sequenase Cy5.5 dye terminator cycle sequencing kit and SEQ4x4 personal sequencer system (Amersham Pharmacia Biotech). A BLAST search against the complete genomic sequence of Nine Mile phase I (24) was achieved to identify the complete nucleotide sequence of the cloned gene. The nucleotide sequence and the deduced amino acid sequence were analyzed by the GENETYX analyzing system (Software Development Co., Ltd., Tokyo, Japan).

**Detection of the *adaA* gene from various isolates of *C. burnetii* by PCR.** A pair of primers, P28F and P28R, was designed based on the *adaA* gene sequence and used to amplify a 269-bp fragment (ranging from positions 369 to 637 in the open reading frame [ORF] region of the *adaA* gene) from DNAs of 17 isolates from various clinical and geographical sources. The sequences of the primers are as follows: P28F, 5'-AATAGATTCGCTCTCTCAAGCCG-3', and P28R, 5'-TCA CCGCTGTTTTTTCAGACG-3'. PCR was performed with 2.5 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, Calif.) in 50  $\mu$ l of reaction mixture containing 20 ng of genomic DNA, 0.2  $\mu$ M (each) primer, and 200  $\mu$ M (each) deoxynucleotide triphosphates in 10 mM Tris-HCl (pH 8.3)–50 mM KCl–2.5 mM  $MgCl_2$ . The reactant was subjected to 35 cycles of 30 s at 94°C, 30 s at 53°C, and 1 min at 72°C in a DNA thermal cycler (PTC-0200 DNA Engine; MJ Research, Inc., Waltham, Mass.).

**Southern blotting.** Restriction enzyme-digested DNAs from various clinical phenotypic isolates including two acute prototypic isolates (Nine Mile and Henzlerling) and five chronic prototypic isolates (Priscilla, Q217, Q229, MAN, and ME) were tested by Southern blotting with the *adaA* gene-specific probe.

**Expression and purification of the *adaA* fusion protein.** The 602-bp DNA fragment of the *adaA* gene was amplified from *C. burnetii* Nine Mile DNA by PCR with primers P28EF-P28ER, which were designed from the *adaA* gene sequence and included 602 bp of the ORF region without the signal peptide-encoding sequence. Primer P28EF (5'-TTCGCTGCCACCGGATCTTC-3') is the 5' end of the *adaA* gene with an additional BamHI restriction site, and primer P28ER (5'-ATCAACTCGAGGTTTCTTCG-3') is complementary to the 3' end of the gene with a XhoI restriction site in the sequence. The amplified *adaA* gene fragment was digested with BamHI and XhoI, ligated to expression vector pET23a, and then transformed into *E. coli* BL21(DE3)LysS competent cells. Expression of T7-tagged (N-terminal) and His-tagged (C-terminal) recombinant *adaA* (*radaA*) was induced by 4 mM IPTG. *radaA* was purified by using a ProBond resin column (Invitrogen) under denaturing conditions.

**Antiserum preparation and immunoblot analysis of *adaA* among various strains of *C. burnetii*.** The anti-*adaA* specific antibody was produced by immunization of BALB/c mice with purified *radaA*. Briefly, BALB/c mice (6 weeks old) were immunized with purified recombinant fusion protein in adjuvant (Titermax) three times at 14-day intervals. At each immunization, mice were subcutaneously injected with 50  $\mu$ l of antigen (containing 20  $\mu$ g of *radaA*) mixture with 50  $\mu$ l of Titermax. After the third immunization, serum was collected and stored at –20°C.

The expression of *adaA* in various strains of *C. burnetii* was confirmed by immunoblotting with anti-*adaA* specific serum. SDS-PAGE and immunoblotting were performed as described elsewhere (31).

**Reactivity of purified *radaA* with infection-derived sera.** The reactivity of *radaA* with sera from guinea pigs infected with various strains of *C. burnetii* was

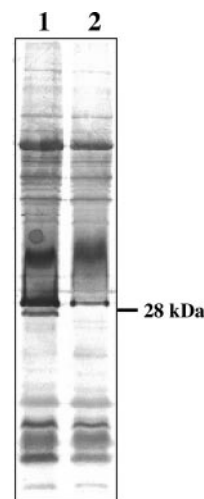


FIG. 1. Immunoblot analysis of purified OMPs of Nine Mile and Q217 strains with rabbit anti-Nine Mile hyperimmune serum. Lane 1, Nine Mile; lane 2, Q217.

analyzed by immunoblotting. Guinea pig serum was collected at 4 weeks post-aerosol infection with  $10^6$  organisms of the Nine Mile phase I, Ohio, Q217, or Q229 strain and stored at –80°C until use. *C. burnetii* Nine Mile whole-cell lysate and purified rCom1, which is a protein common to all isolates tested (29, 30), were used as a control to confirm the presence of the antibodies to *C. burnetii* antigens in infection-derived sera. SDS-PAGE and immunoblotting were performed as described previously (31).

## RESULTS

**Cloning the *adaA* gene.** Immunoblotting identified an immunoreactive band at 28 kDa in the purified membrane fraction of *C. burnetii* Nine Mile but did not detect reactivity in the Q217 strain (Fig. 1). The result confirmed that the 28-kDa protein corresponds to the *adaA* previously noted (6). To identify the *adaA* gene, we determined the N-terminal 31 amino acids of a 28-kDa protein from *C. burnetii* Nine Mile. Based on the amino acid sequence, we designed several primer pairs and successfully amplified a 59-bp fragment from *C. burnetii* Nine Mile DNA by PCR. The 59-bp fragment was used as a DNA probe to screen a genomic library of *C. burnetii* Nine Mile DNA. Approximately 10,000 plaques were screened by Southern hybridization with the *adaA* gene-specific probe. Forty positive plaques were purified and compared for expression of immunoreactive proteins. Coomassie brilliant blue (CBB) staining on an SDS-polyacrylamide gel identified one clone, designated p110, expressed as an ~24-kDa protein. Immunoblotting indicates that the protein expressed by clone p110 reacted with rabbit anti-Nine Mile serum (data not shown), suggesting that the clone p110-expressed recombinant protein is specific for *C. burnetii*.

**Sequence analysis.** To determine the nucleotide sequence of the ORF encoding *adaA*, the purified recombinant plasmid from clone p110 was sequenced. The *adaA* gene-specific primers P28a1 and P28b1 were used in a sequence reaction to directly determine the nucleotide sequence of the *adaA* gene. The sequence of the p110 cloned insert was BLAST searched against the complete genome sequence of *C. burnetii* Nine Mile RSA493, allowing confirmation of the nucleotide sequence of

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1  cgctcttcagttaccctaaagccgatcgctctttatccgcggatatttcctgttggtgggctaataagcttgc 70
71  ttatttcttgccttttgggtgcccctgatgatttgggtcaattggcataggcctgattgctgttggacttg 140
141  cttggaatatattttatggctcgtttatggatatgagcaattttgaataggtggtgtatttatcagcaat 210
      -35
211  gttatgctaataaggaactgctgttctagcgcaatagaagaggggtcacATGAAAACTAACCGT 280
      -10      RBS      M K K L T V
281  AACTTTCTTAACCTTTATTAGTATTTCTTCGCTGCCACCGCAGCTTCGCTGAAAAATCGCCCATTTTA 350
      T F L T F I S I F F A A T A A F A E N R P I L
      Signal peptide
351  AATACGATTAATTATCAACAGCAAGTTGAAAAATGGGTAACGACGGATTCTGCCGATGTGATGTTCCG 420
      N T T N Y O O O V F K W V T T D S A D V M V S V
      Chemically determined N-terminal 31 amino acids of the P28
421  TTAACGTTACAACAAAAGAAAAAGTTTGATGCACTGCAACACCAAGTAATGAAAAATGGAAGAATT 490
      N V T T K E K K F D A L Q H Q V M K K L E E L
491  AAGTGCAGGACGTCAATGGCATATCGATTCTTTTCAATGTCACAGGATCAGTCGGGATTGGAAGTTTAA 560
      S D G R Q W H I D S F S M S Q D Q S G L E V L
561  AGCTGGGAAGTCCGCTCTCGAATGCCTTTGGCTTGGTGAACAGTCTGCGTCAAAAAATAGATTGCTCT 630
      S W E V R S R M P L A L V N S L R Q K I D S L S
631  CTCAAGCCGGACAACATATAAAATCAGAACGTTGATTTTGAACCTAGTTTAGTAGAAAAAGAAAAAGC 700
      Q A G Q Q Y K I Q N V D F E P S L V E K E K A
701  TTTGCTGAGCTGCCAGCGAGTTTACGATCAAGTTAAAGTAGAGTTAGATAATTTGAATAAATCGTTT 770
      F A E L R Q R V Y D Q V K V E L D N L N K S F
771  CCGAATGGTCATTATTTTACATAGCATCGATTGTCTCTCCACCGCTCTACGCCCTAACCAAAAAG 840
      P N G H Y F L H S I D F V S P P L Y A A N Q K E
841  AATTAACGCTAATGCGATCTGCCCCGTCTGAAAAACAGCGGTGACTTTGGGAAGAAACCTAATGTTGAT 910
      L T L M R S A P S E K T A V T L G R N L M L I
911  TGCGAATGTAAAAGTGGGACACTTTTTAAATAAATAGGcaataacgctagaaaagttcatactattttt 980
      A N V K V A T F L N K *
981  ttacaaaacttttctaattttataagaatagaaaaagggctttgtcattgaagcattcaatccatctg 1050
1051  atttataaaactattttcattccaagcacgccaatatttattatttagaacattgtcgtatccttgttaa 1120
1121  tggcggctgagtagagtatgtcactcaggaaggtagccagtcgtgtgtattggaatatccctattgcaaat 1190
1191  accacaacaattttattggggacgggaacttcggttactcaagcagccat 1240

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FIG. 2. Nucleotide sequence of the *adaA* gene. The deduced amino acid sequence is shown beneath the nucleotide sequence of the ORF. A putative promoter (positions -35 and -10) is underlined (thin line). The predicted ribosome-binding site (RBS) is underlined (thick line). The 20-amino-acid sequence at the N-terminal end of *adaA* is predicted to be a signal peptide. The chemically determined N-terminal 31-amino-acid sequence was identical to the N-terminal end of the deduced amino acid sequence of *adaA*.

the cloned gene (24). The nucleotide sequence (1,240 bp) from the cloned gene is shown in Fig. 2, which includes the flanking regions and the deduced amino acid sequence of P28. The *adaA* gene has a predicted ORF consisting of 684 bp, starting with an ATG codon at position 264 and ending with a TAG codon at position 947. The ORF is preceded by a putative ribosome-binding site, GGAGG, from 7 bp upstream of the ATG start codon. A predicted promoter sequence, TTGAAT-21 nt-TGTTAT, was found 36 bp upstream from the putative ribosome-binding site. The G+C content of the ORF coding region was 40%, which is similar to the value of *C. burnetii* total genomic G+C content (43%). The predicted mature P28 protein consists of 227 deduced amino acid residues and has a calculated molecular mass of 25,950 Da and a theoretical isoelectric point of 8.60. A 20-amino-acid signal

peptide was also predicted for the N-terminal sequence of *adaA*. We also confirmed that the chemically determined N-terminal 31 amino acids of *adaA* were identical to the amino acid sequence of the mature protein deduced from the nucleotide sequence of the cloned *adaA* gene, which is the region immediately adjacent to a predicted signal peptide (Fig. 2). In addition, a BLAST search of GenBank with the deduced amino acid sequence of the *adaA* gene indicated that the ORF encoding *adaA* was identical to CBU0952, which was predicted by The Institute for Genomic Research gene annotation (24). These results suggest that the ORF identified in the p110 cloned gene sequence is a gene unique to *C. burnetii* and encodes *adaA*.

**Detection of the *adaA* gene in various isolates of *C. burnetii*.** PCR and Southern blotting were used to test and confirm



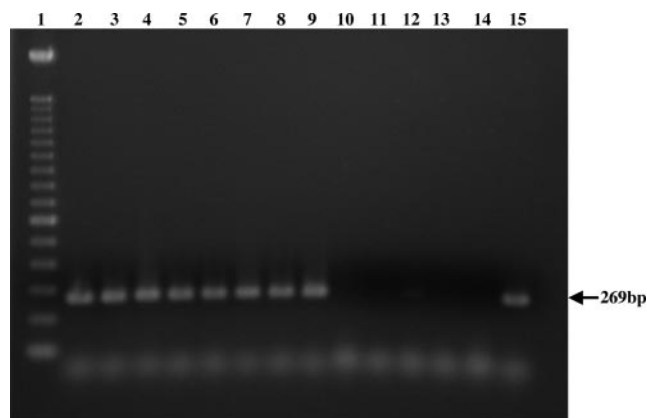


FIG. 3. Detection of the *adaA* gene from various isolates of *C. burnetii* by PCR with primers P28F-P28R. Shown is an ethidium bromide-stained agarose gel electrophoretogram of PCR-amplified products. Lane 1, molecular size markers (100-bp DNA ladder); lanes 2 to 9, isolates originating from ticks, milk, and humans with acute Q fever (Nine Mile, Ohio, California, El Tayeb, Africa, Panama, Turkey, and Giround, respectively); lanes 10 to 14, isolates originating from a goat and humans with chronic Q fever (Priscilla, KQ154, KoQ229, SQ217, and GQ212, respectively); lane 15, Dugway isolate.

whether the *adaA* gene is unique for a subgroup of isolates. The PCR result indicated that the *adaA* gene-specific fragment was amplified from isolates originating from humans with acute Q fever, ticks, cattle, and rodents, but PCR did not amplify any product from isolates from goats or humans with chronic Q fever (Fig. 3). Southern blotting also indicated that the *adaA* gene-specific probe hybridized with one band with *Sal*I-digested DNAs of Nine Mile and Henzerling strains associated with acute Q fever but did not hybridize with any band with *Sal*I-digested DNAs of Priscilla, Q217, Q229, MAN, and ME strains, which have been linked to chronic Q fever (data not shown). These results suggest that the *adaA* gene is specific for *C. burnetii* isolates originating from humans with acute Q fever, ticks, cattle, and rodents.

**Expression of *adaA* in *E. coli* and various isolates of *C. burnetii*.** The partial *adaA* protein of the Nine Mile strain was overexpressed as a fusion protein in pET23a. An IPTG-inducible fusion protein with a molecular mass of 28 kDa was detected in the *adaA* gene recombinant pET23a-transformed *E. coli* culture by CBB staining of the SDS-polyacrylamide gel and immunoblotting with a His-tagged specific monoclonal antibody (Fig. 4A and B, lanes 2 and 3). The expressed fusion protein was not detected in the negative control of pET23a-transformed *E. coli* culture (Fig. 4A and B, lanes 1). SDS-PAGE and immunoblotting also indicated that *radaA* was successfully purified from the *adaA* gene recombinant pET23a-transformed *E. coli* culture by using ProBond resin column (Fig. 4A and B, lanes 4). To confirm that P28 is expressed by acute disease isolates but not carried by chronic disease isolates, anti-*radaA* specific antibody was produced and used in immunoblotting with antigens of various strains of *C. burnetii*. Immunoblotting indicated that a ~28-kDa reaction band was detected from acute-disease-associated isolates Nine Mile and Henzerling but not observed in chronic-disease-associated isolates Priscilla and Q217 (Fig. 4C). This result confirmed that our cloned *adaA* gene encodes *adaA* and that *adaA* is ex-

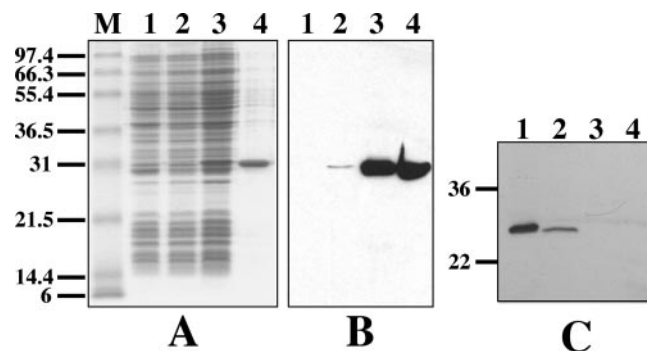


FIG. 4. Expression of the *adaA* gene in the pET23a expression vector system. Expression of the T7-tagged (N-terminal) and His-tagged (C-terminal) fusion protein was monitored by CBB staining of uninduced and induced cultures by SDS-PAGE and immunoblotting with His-tag-specific monoclonal antibody. (A) CBB staining profile of the expressed and purified fusion *adaA* protein. Lane M, molecular size markers; lane 1, pET23a-transformed *E. coli* (negative control); lanes 2 and 3, *adaA* gene recombinant pET23a-transformed *E. coli* uninduced and induced cultures, respectively; lane 4, purified fusion *radaA*. (B) Immunoblot analysis of expressed and purified recombinant proteins with His-tag-specific monoclonal antibody. The samples shown in panel B are the same as in panel A. (C) Immunoblot analysis of *adaA* in various strains of *C. burnetii* with *adaA*-specific antiserum. Lanes 1 to 4, whole-cell lysate of Nine Mile, Henzerling, Priscilla, and Q217 strains, respectively. A ~28-kDa reaction band was observed in acute disease isolates including the Nine Mile and Henzerling strains but not detected in Priscilla and Q217. Numbers at left are molecular masses in kilodaltons.

pressed by acute-disease-associated isolates but not carried by chronic-disease-associated isolates.

**Reactivity of purified *radaA* with sera derived from infected animals.** Figure 5 shows the immunoblots of the whole-cell antigen, rCom1, and *radaA* with sera derived from a guinea pig

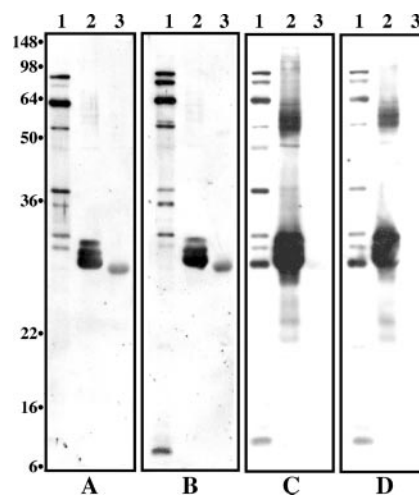


FIG. 5. Immunoblots of whole-cell antigen, rCom1, and *radaA* with sera derived from guinea pigs 4 weeks post-aerosol infection with  $10^6$  organisms of either the acute or the chronic prototypic isolate of *C. burnetii*. Sera (diluted 1:500) came from guinea pigs infected with Nine Mile (A), Ohio (B), Q217 (C), and Q229 (D). The samples shown in panels A to D are the same. Lanes 1, Nine Mile whole-cell antigen; lanes 2, purified rCom1 protein; lanes 3, purified recombinant *adaA* protein. Numbers at left are molecular masses in kilodaltons.

4 weeks post-aerosol infection with  $10^6$  organisms of the acute or chronic prototypic strain of *C. burnetii*. All sera from guinea pigs aerosol infected with  $10^6$  organisms of various strains strongly reacted with Nine Mile whole-cell antigen at a wide range of molecular weights and with rCom1 at similar levels (Fig. 5, lanes 1 and 2). However, *radaA* reacted with sera from guinea pigs infected with isolates Nine Mile and Ohio (Fig. 5A and B, lanes 3) but not with sera from those infected with isolates Q217 and Q229 (Fig. 5C and D, lanes 3). These results indicate that anti-*adaA* specific antibody was present in sera derived from animals infected with the acute prototypic isolate but absent in sera from animals infected with the chronic prototypic isolate.

## DISCUSSION

Cloning and characterization of *adaA* demonstrated that this protein is specific for acute-Q-fever-related isolates but deleted in chronic-disease-associated isolates despite geographical source, suggesting that *adaA* may be a virulence factor involved in the pathogenesis of acute Q fever in humans.

The predicted *adaA* mature protein consists of 227 amino acids and has a predicted molecular mass of 25,950 Da. This is very close to the molecular size of native *adaA* expressed in *C. burnetii* but about 2 kDa larger than the expression product of the *adaA* gene in *E. coli* (data not shown). The 25-amino-acid signal peptide is predicted in the N-terminal sequence of *adaA*, which is probably cleaved from the mature protein when the *adaA* gene is expressed in *E. coli*. The chemically determined N-terminal and internal peptide (data not show) amino acid sequences of *adaA* were identical to the deduced amino acid sequence of the cloned *adaA* gene, confirming that the identified ORF encodes *adaA*. The cloned *adaA* gene recombinant pUC19 expressed *radaA* in *E. coli* DH5 $\alpha$  cells without induction by IPTG (data not shown). A potential promoter sequence, TTGAAT-21 nt-TGTTAT, was identified in the *adaA* gene sequence, suggesting that the *adaA* gene was expressed in *E. coli* by using the endogenous promoter. A BLAST search of GenBank with either the nucleotide sequence or the deduced amino acid sequence for the *adaA* gene did not identify significant DNA or amino acid homologies, suggesting that *adaA* is unique to *C. burnetii*.

OMPs of gram-negative bacteria are employed in several important roles in the host-parasite interaction and relate to both pathogenesis and protective immunity. Due to the difficulties in cultivation and purification of *C. burnetii*, only a limited group of OMP-encoding genes have been characterized (4, 16, 28). Candidates for OMPs include the QpH1 plasmid-specific gene *cbhE'* for a 42-kDa surface protein (15) and the QpRS plasmid-specific gene *cbbE'* for a 55-kDa surface protein (12–14), which have been speculated to be virulence related and associated with acute or chronic Q fever in humans. However, recent investigations of several European isolates suggested that there were no specific genes on plasmids responsible for acute or chronic Q fever (25–27) and supported the notion that host factors may play a key role in the development of chronic Q fever. It remains unknown whether there are specific genes on the chromosome responsible for acute or chronic Q fever. Isolates from acute disease are distinct from chronic-disease-associated isolates at the molecular level (3, 5,

22) and in a guinea pig fever model of acute disease (17; K. Russell, unpublished data), suggesting different virulence potentials for groups of isolates of *C. burnetii*. In this study, we identified a novel ~28-kDa membrane-associated protein and demonstrated that *adaA* is expressed in acute group isolates but not carried by chronic group isolates, suggesting that *adaA* may be a virulence factor related to acute Q fever. Immunoblotting with purified *radaA* antigen recognized anti-*adaA* specific antibody from sera derived from animals infected with acute group isolates but not from sera from animals infected with chronic group isolates, suggesting that *adaA* is an important antigen in acute disease. Since there has been no suitable animal model developed to represent the manifestation of chronic Q fever and because there is a lack of genetic tools for *C. burnetii*, it is not possible to directly test whether a specific gene is related to acute or chronic disease. Recently, SCID mice have been used as a model highly sensitive to lethal challenge by an acute-disease-associated isolate of *C. burnetii* (1), and preliminary comparison in this model shows dramatic differences in disease from isolates which do not carry *adaA* (M. Andoh, unpublished data). Further studies to test whether the *adaA* gene can be delivered on a stable plasmid to a *adaA*-negative isolate may allow its role in virulence to be determined.

Since prompt antibiotic therapy could lead to a better prognosis for individual patients with chronic Q fever, developing a diagnostic method for rapid differential diagnosis of acute and chronic Q fever could be very important for control of chronic disease. Recently, based on point mutations unique to isolate groups, *com1* and *icd* genes have been used as genetic markers to distinguish acute and chronic isolates (18, 29). However, comparison of nucleotide sequences of *com1* and *icd* genes among isolates indicates that they are highly conserved between acute and chronic isolates, except for these few point mutations (18, 29). The finding that the *adaA* protein and the *adaA* gene are unique to acute group isolates can be used for development of *radaA* antigen-based serodiagnostic methods and/or an *adaA* gene-targeted PCR assay for differential diagnosis of acute and chronic Q fever in clinical samples. We have designed primers based on the nucleotide sequence of the *adaA* gene and used them to amplify products from DNA of various strains of *C. burnetii*. Amplicon products were amplified from DNA templates of isolates originating from humans with acute Q fever, ticks, cattle, and rodents but not from isolates originating from humans with chronic Q fever, suggesting that PCR for the *adaA* gene can be used for differentiation of acute- and chronic-disease-associated isolates. In addition, immunoblotting indicated that *radaA* reacted with sera derived from animals infected with acute group isolates but was not recognized by sera derived from animals infected with chronic group isolates, suggesting that an *radaA* antigen-based serodiagnostic test may be useful for differential diagnosis of acute and chronic Q fever in human sera. Further studies will evaluate the usefulness of an *adaA* gene-targeted PCR assay and an *radaA* antigen-based enzyme-linked immunosorbent assay for differential diagnosis of acute and chronic Q fever in clinical samples from acute and chronic Q fever patients.

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